ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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OPEN ACCESS International Journal of Poultry Science

ISSN 1682-8356
DOI: 10.3923/ijps.2018.467.472

Research Article
Using Prophylactic *Salmonella* Immune Lymphokines to Resist the Avian Influenza in Broiler Chickens

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Abstract

Objective: The current study aimed to increase the resistance of broiler chickens against Avian Influenza Virus (AIV) type H5N8. Early infections with AIV are caused by weak immunity, whether maternal or acquired immunity from vaccinations and all the AIV vaccines are killed vaccines that provide absolute protection after three weeks of vaccination, which makes the chicks susceptible to infection at an early age. Our goal is to enhance the immune resistance against avian influenza at an early age by using immune lymphokines from hyperimmunized chickens with *Salmonella enteriditis*. **Materials and Methods:** The experiment was carried out on 250 broiler chicks divided into five groups, with 50 chicks per group. All groups were treated at the first day as follows: G1: injected with *Salmonella*-immune lymphokines (S-ILK) intraperitoneally after a 30 min intramuscular challenge with AIV (H5N8). G2: *Salmonella*-nonimmune lymphokines (S-NILK) were injected intraperitoneally after a 30 min intramuscular challenge with AIV (H5N8). G3: injected with S-ILK without a challenge with AIV (H5N8). G4: only challenged with AIV (H5N8) and considered a positive control group. G5: not treated and not challenged and considered a negative control group. **Results:** The results of the present study showed a significant increase ($p<0.05$) in the antibody titre in all treated groups, except the G3 and G5 groups, which were not challenged. The G1 group recorded a moderate increase in antibodies with the lowest mortality rate, followed by the G2 group, compared to the G4 group, which recorded a 100% mortality rate on day 28 after challenge. The results of the viral load revealed the highest number of the influenza RNA copies in the G4 group compared with the G1 group, which recorded the lowest number of RNA copies that did not pose a serious risk to the lives of infected birds, followed by G2. **Conclusion:** Giving S-ILK at early ages increases the immune resistance against avian influenza (H5N8), which in turn compensates for live vaccines at early ages.

Key words: *Salmonella enteriditis*, immune lymphokines, avian influenza, ELISA, viral load

Received: July 16, 2018 Accepted: August 24, 2018 Published: September 15, 2018

Citation: Mushtaq T.B. AL-Zuhariy, 2018. Using Prophylactic *Salmonella* Immune Lymphokines to Resist the Avian Influenza in Broiler Chickens Int. J. Poult. Sci., 17: 467-472.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) is one of the worst viral contagious diseases and is devastating in poultry, cosmopolitan in occurrence and poses a potential pandemic threat to humans[1]. Broiler and layer chickens of all ages can be infected and the variable mortality rate ranges from 30-100% depending on virus pathogenicity. Avian Influenza Virus (AIV) is considered among the diseases that have significant potential for destruction in commercial poultry production, which cause huge economic losses, especially in breeder and layer flocks[2]. Avian and mammalian influenza viruses belong to the orthomyxoviridae family, which consists of four types (A, B, C and D) and all influenza infections (avian, equine, porcine and humans) are caused by type A influenza viruses[3]. Influenza Type A viruses are roughly filamentous or spherical particles, 80-120 nm in diameter. The nucleocapsid consists of a helical symmetry and is coated with matrix protein; the surface is covered with two types of glycoproteins (16 Hemagglutinin and 9 Neuraminidase)[4]. The positive effects of cytokines on the pathogenesis of influenza virus (HPAI) are variable depending on the strains, hosts and stages of infection[4,5]. In studies carried out on mice infected with influenza, an excessive inflammatory response and cytokine dysregulation was observed, resulting in high mortality[6]. The regulation of cytokine production from phagocytic cells activated the high and rapid proliferation of T cells and natural killer cells to reduce viral infection and apoptosis. In addition, other studies on mice infected with influenza H5HPAI found high mortality due to lack of mouse interleukin (IL-6) and TNF[7]. The rapid onset of the disease is due to the weak immune response of the host and the absence of host-virus interaction, which occurs in flocks infected with HPAI influenza. Kuribayashi *et al.*[8] verified that the weakness of inflammatory responses and cytokines of T-helper 1 (Th1) after infection with H5N1HPAI in birds with weak immune resistance causes the rapid and extensive proliferation of the virus with the generation of a fatal inflammatory response in birds without reducing the virus proliferation. However, in acute cases of HPAI H5N8 infection, any increase in cytokine gene expression in lungs was not observed until the death of infected birds. Therefore, vaccination with a killed vaccine in early ages to prevent HPAI infections did not work unless there was an enhancement of primary response before infection because the killed vaccines were slow to generate absolute protection, in addition to the genetic changes of the influenza virus from time to time[9]. This study was conducted to show the positive effects of *Salmonella*-immune lymphokines to increase the immune resistance in broiler chickens against HPAI.

MATERIALS AND METHODS

Lymphokines preparation: An unpublished isolate of *Salmonella enteritidis* was taken from a section of Poultry Diseases-Department of Diseases and Poultry Diseases-College of Veterinary Medicine - University of Baghdad. The isolate was grown by using nutrient broth and peptone water and incubated at 37°C for 24 h until it developed turbines and small white sediment was observed. Then, a sample of sediment was grown on *Salmonella*-selective media such as Macconkey agar and SS agar. API20 was used for biochemical identification. After confirmation of the type of *Salmonella* isolate, a suitable bacteria solution was prepared at a concentration of 1×10^8 colony-forming unite/mL based on spectrophotometric data to determine the desired concentration by using the standard curve of 625 nm in length.

Then, two groups of one-day-old broiler chicks were raised at the Animal House of the College of Veterinary Medicine-University of Baghdad. The first group was vaccinated with three doses of *Salmonella enteritidis* (1 mL/bird, orally) at 7, 14 and 21 days, while the second group was orally administered a saline solution and was considered a negative control group. On day 30, spleens were collected from the vaccinated and unvaccinated chicks with *Salmonella enteritidis* after slaughtering and sent to the laboratory to prepare S-ILK from T cells of vaccinated chicks and to prepare S-NILK from T cells of unvaccinated chicks according to the method described by Reed and Muench[10].

Preparation of the viral inoculum: The unpublished isolate of the influenza virus H5N8 (allantoic fluid) was taken from the section of Poultry Diseases-Department of Pathology and Poultry Diseases-College of Veterinary Medicine-University of Baghdad. ELD_{50} was identified as $10^{8.5}$ by Hemagglutination test and used to challenge in $100ELD_{50}$ $10^{6.5}$ according to the method of Reed and Muench[10]. The sample was stored in a deep freeze (-80°C) until use.

Experimental design: The experiment was carried out on 250 broiler chicks divided into five groups, with 50 chicks per group. All groups were treated at the first day as follows: G1: injected with *Salmonella*-immune lymphokines (S-ILK) (0.5 mL/chick) in the intraperitoneal, after a 30 min intramuscular challenge with AIV (H5N8) ($100ELD_{50}$ $10^{6.5}$). G2: *Salmonella* nonimmune lymphokines (S-NILK) (0.5 mL/chick) were injected in the intraperitoneal, after a 30 min intramuscular challenge with AIV (H5N8) ($100ELD_{50}$ $10^{6.5}$). G3: injected with S-ILK (0.5 mL/chick) without challenge

with AIV (H5N8). G4: only challenged with AIV (H5N8) ($100ELD_{50}$ $10^{6.5}$) and considered the positive control group. G5: not treated and not challenged and considered the negative control group. Clinical signs, pathological changes and mortality rates were recorded after the challenge until the end of the experiment.

Sampling: Five blood samples were collected from each group from the right jugular vein at 7, 14, 21 and 28 days. Glass tubes free of anticoagulant were used to separate the serum from the blood samples using a centrifuge at 1000 rpm for 15 min. Additionally, five samples of lung tissue were collected for each group at 7, 14, 21 and 28 days and stored in deep freeze (-80°C). RT-PCR was used to calculate the RNA copies of the HPAI H5N8 virus.

Morbidity and mortality: Clinical signs were recorded for the surviving birds from pre-acute infection during the experimental period. Respiratory signs included coughing, sneezing, whistling and rales were recorded. Other signs included neurological developments resulting from CNS infection, such as torticollis, opisthotonos, paralysis, in coordination and drooping wings were recorded. Pathogenic lesions were recorded during the experimental period, such as skin lesions, including facial swelling, cyanosis in comb and wattles, edema and red discoloration of the legs and feet as a result of subcutaneous ecchymotic bleeding; visceral lesions included petechial hemorrhage in internal organs and muscles, nose and mouth colored with blood and in acute infections, green diarrhea was observed.

Real time (RT-PCR): By using this test, the number of RNA copies of the influenza virus HPAI H5N8 was calculated in lung tissues and the results were analyzed according to Sun *et al.*[11]. RNA was extracted from the lung tissue of infected birds by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Special primers and TaqMan probes were used against AIV RNA for the detection of influenza viruses. A real-time PCR test was performed depending on the LightCyler® 480 real-time PCR system (Roche Diagnostic Deutschland GMbh, Mannheim, Germany) by using a single-step Prime Script RT-PCR kit[11].

ELISA test: Antibodies of H5N8 were detected in the serum of infected birds by using the ELISA test, which is one of the most important serological tests, characterized by high sensitivity and specificity in the detection of serum immune antibodies; the ELISA kit used was manufactured by the company ProFlock AIV. The procedure was carried out in accordance with the method described by Synbiotic® Corporation[12].

Statistical analysis: The data were analyzed using Statistical Analysis System (SAS). Means were separated by the least significant difference (LSD) test. Statistical significance was set at $p<0.05$.

RESULTS

Immunity against avian influenza: The results of the ELISA for 10 serum samples, randomly collected on the first day from 250 chicks before they were divided into groups, showed good maternal immunity (8365±225). The results showed a significant increase ($p<0.05$) in the antibody titre in treated groups in comparison with the negative control group (G5) during the experimental period. The group G1 showed a significant increase ($p<0.05$) in the antibody titre at 14 and 21 days (5643±211 and 7213.3±231.3), followed by the G2 group (3111±312 and 4321.5±342.2), compared to other groups. The G4 group showed a highly significant increase ($p<0.05$) in antibody titre (27654.5±1765.2), followed by G2 (18654.2±964.6), compared with G1, which recorded a moderate increase (9546.6±754) compared to G3 and G5, which did not record any significant changes because they were not challenged with HPAI H5N8 (Table 1).

Table 2 shows the results of the viral load by using RT-PCR after the challenge with H5N8 HPAI on the first day and the G4 group showed a highly significant increase ($p<0.05$) in

Table 1: The antibody titre (Mean±SE) against AIV by ELISA in broiler chickens after being challenged with H5N8 (100 ELD50 $10^{6.5}$)

	AIsV antibody titre Means±Standard error			
Periods	7 days	14 days	21 days	28 days
Groups				
G1	3245.4±244.2^{B}	5643±211^{A}	7213.3±231.3^{A}	9546.6±754^{C}
G2	22265±233.4^{C}	3111±312^{B}	4321.5±342.2^{B}	18654.2±964.6^{B}
G3	6213.1±227.1^{A}	2100±146.9^{C}	0±0^{C}	0±0^{D}
G4	2134.2±213.3^{C}	0±0^{D}	0±0^{C}	27654.5±1765.2^{A}
G5	6532.1±321.5^{A}	2001±188.5^{C}	0±0^{C}	0±0^{D}
LSD	722	354.2	432.6	2432.4

No. of samples: 5 from each group. The large letters indicate significant differences ($p<0.05$) between the treatment means. LSD: Least significant difference

Table 2: The number of RNA copies of H5N8 (mean±SE) in lung tissue by RT-PCR in broiler chickens after the challenge of H5N8 (100 ELD50 $10^{6.5}$)

	Viral load Means±Standard error			
Periods	7 days	14 days	21 days	28 days
Groups				
G1	675±22.4^{C}	1234.6±65.4^{C}	2897.5±233.8^{C}	3022.6±185.5^{C}
G2	2345±33.5^{B}	3675.2±123.6^{B}	5675.6±254^{B}	7896.6±324^{B}
G3	0±0^{D}	0±0^{D}	0±0^{D}	0±0^{D}
G4	3654.3±176.5^{A}	5674±211.3^{A}	8756±897.2^{A}	11342.3±432^{A}
G5	0±0^{D}	0±0^{D}	0±0^{D}	0±0^{D}
LSD	287.2	678.7	965.5	1575.3

No. of samples: 5 from each group. The large letters indicate significant differences ($p<0.05$) between the treatment means. LSD: Least significant difference

Table 3: The clinical signs of treatment groups in different periods during the experiment

	Groups				
Signs	G1	G2	G3	G4	G5
Respiratory	15/50 (30%)^{C}	40/50 (80%)^{B}	0/50 (0%)^{D}	50/50 (100%)^{A}	0/50 (0%)^{D}
Others	10/50 (20%)^{C}	30/50 (60%)^{B}	0/50 (0%)^{D}	50/50 (100%)^{A}	0/50 (0%)^{D}
Mortality	5/50 (10%)^{B}	50/50 (100%)^{A}	0/50 (0%)^{B}	50/50 (100%)^{A}	0/50 (0%)^{B}

Respiratory signs include coughing, sneezing, rales and whistle, Others include the development of CNS involvement evident as torticollis, opisthotonos, incoordination, paralysis and drooping wings

Table 4: The pathogenic lesions at different periods in the treatment groups during the experiment

	Groups				
Lesions	G1	G2	G3	G4	G5
Cutaneous	15/50 (30%)^{C}	27/50 (54%)^{B}	0/50 (0%)^{C}	50/50 (100%)^{A}	0/50 (0%)^{C}
Visceral	10/50 (20%)^{C}	30/50 (60%)^{B}	0/50 (0%)^{C}	50/50 (100%)^{A}	0/50 (0%)^{C}
Others	17/50 (34%)^{C}	35/50 (70%)^{B}	0/50 (0%)^{C}	50/50 (100%)^{A}	0/50 (0%)^{C}

Cutaneous lesions include facial swelling, cyanosis in comb and wattles, edema and red discoloration of the legs and feet as a result of subcutaneous ecchymotic bleeding, Visceral lesions include petechial hemorrhage in internal organs and muscles, Others include nose and mouth colored with blood and finally, in acute infections, green diarrhea was observed

number of RNA copies of the influenza virus in the lung tissue at 7, 14, 21 and 28 days. This was followed by the G2 group, while the G1 group showed the lowest level ($p<0.05$) in the number of RNA copies of HP5 H8N8, compared with the G3 and G5 groups, which did not record any numbers of RNA copies of the influenza virus.

Clinical signs and mortality: Table 3 shows the clinical signs that were recorded for birds surviving the pre-acute infection during the experimental period. Respiratory signs included coughing, sneezing, whistling and rales. Other signs included neurological developments resulting from CNS infection, such as torticollis, opisthotonos, paralysis, in coordination and drooping wings. The G4 group recorded the highest incidence of clinical signs in all infected birds and had the highest mortality rate of 100%, followed by G2, which recorded a moderate percentage, while G1 recorded the lowest morbidity and mortality in birds challenged with H5N8 HPAI, as compared with G3 and G5, which reported no infections.

Table 4 shows the gross lesions that were recorded during the experimental period, such as skin lesions, including facial swelling, cyanosis in comb and wattles, edema and red discoloration of the legs and feet as a result of subcutaneous ecchymotic bleeding; visceral lesions included petechial hemorrhage in internal organs and muscles, nose and mouth colored with blood; and finally, green diarrhea was observed in acute infections. The G4 group recorded the highest percentage of lesions in all infected birds, followed by G2, which recorded a moderate percentage, while G1 recorded the lowest percentage of lesions in birds challenged with H5N8 HPAI as compared with G3 and G5, which reported no infections.

DISCUSSION

The chicks were protected depending on maternal immunity, which is acquired from breeders vaccinated with oil killed vaccine. Maternal immunity, which was recorded at the highest level in the first days after hatching in broiler chickens, begins to decline gradually to zero at week 2 or 3[13]. In the present study, S-ILK enhanced maternal immunity and increased resistance against HPAI H5N8

infection. De Vriese *et al.*[14] has shown that maternal immunity protects the chicks in the first week only and they remain susceptible to AIV in the second week, particularly to HPAI infections. Broiler chickens from vaccinated mothers with high maternal immunity challenged with HPAI H5N8 in 11 days resulted in an estimated 83% mortality rate. The results of the ELISA agree with Mass *et al.*[15], who demonstrated that being vaccinated with repeated doses of vaccines generate cumulative immunity transmitted to chicks through eggs, protect flocks from HPAI in the first week and may last for more than 10 days. Wong *et al.*[16] reported that high levels of maternal immunity in the early days begin to decline gradually, decrease to half after five days but fall to zero after 7 days due to challenges with the influenza virus. The results of the current study agree with Van der Goot *et al.*[17] who found a highly significant reduction in the level of maternal immunity after the challenge due to neutralization between the virus and maternal immunity, even with a weak virus in attenuated vaccines. The current study showed the biological role of lymphokines derived from hyperimmunized birds with *Salmonella typhimurium* in providing highly effective and potent immunity against the challenge of HPAI at early ages. These results agree with Baumgarth *et al.*[18] who demonstrated that the effective response of Th2 cells to cytokines produced from activated phagocytic cells in mice helps reduce virus proliferation in lung tissue.

Similarly, Mass *et al.*[15] demonstrated the significant pivotal role of IL-4 in the reduction of HPAI compared to cellular immunity (IFN-γ), which is reflected by the significant reduction in viral shedding, regardless of the high pathogenicity and proliferation of the virus. The results were consistent with Arai *et al.*[19], who demonstrated the role of lymphocytes in regulating the host immune response, as well as signaling between defensive cells, through the production of cytokines, which are soluble particles produced from many cells, including T, B, macrophage and dendritic cells. In addition, the cytokines have active role to regulate the immune cells during innate or acquired responses as well as during development and homeostasis[19]. Additionally, Moulin *et al.*[20] highlighted the essential role of S-ILK against many viral infections during the development and recruitment of lymphocytes and rapid aggregation in many inflammatory areas. These lymphocytes aggregation play a role in preventing virus proliferation, act to modify and determine the nature of humoral immune response after challenge with HPAI H5N8 infection. Asif *et al.*[21] reported that several recent studies related to avian lymphokines have identified a number of lymphokines that possess antiviral properties against many viral infections and exert an immunomodulatory effect. Collisson *et al.*[22] also noted the importance of IFN-α, which belong to the first type of the interferon family, is produced by cells after viral infections and inhibits the localization of the virus by preventing its reproduction, as well as the role of IFN-γ in preventing the HPAI H5N8 infection, which is produced by CD4 T and natural killer cells, stimulates and proliferates CD8 T cells and cytotoxic T lymphocytes (CTL) to remove viral infection[18]. The G1 group, which was treated with S-ILK and challenged with H5N8 HPAI, recorded the lowest virus proliferation at 7, 14, 21 and 28 days compared to the G2 group treated with S-NILK, which recorded the highest virus proliferation at 21 and 28 days after the challenge. This proved the essential role of S-ILK in reducing the systemic viral load of the H5N8 virus in infected tissues.

CONCLUSION

Salmonella-immune lymphokines play an effective role in increasing the resistance against HPAI H5N8 by enhancing the immune response of infected birds and preventing virus replication in infected tissues after the challenge with HPAI.

SIGNIFICANCE STATEMENT

The current study discovered the role of poultry immune lymphokines to reduce the negative effects of HPAI (H5N8) in broiler chickens by increasing the immune resistance of infected birds and reducing viral reproduction in infected tissues. This study will help researchers uncover the effective role of S-ILK in reducing AVI infections, as well as the effective role of subclinical salmonella infections in early ages in the generation of immunity against many viral infections that many researchers could not explore. Therefore, a new theory has been reached on the use of S-ILK to enhance immunity against severe respiratory infections, such as Newcastle and infectious bronchitis and finally, influenza, so that commercial vaccines, whether live, attenuated and killed, can be dispensed that do not provide absolute protection against severe infections, the genetic mutations of some viral isolates, as well as the effort and cost involved in their application.

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